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REVIEW ARTICLE

Neurovascular coupling mechanisms in health and neurovascular uncoupling in Alzheimer's disease

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To match the metabolic demands of the brain, mechanisms have evolved to couple neuronal activity to vasodilation, thus increasing local cerebral blood flow and delivery of oxygen and glucose to active neurons. Rather than relying on metabolic feedback signals such as the consumption of oxygen or glucose, the main signalling pathways rely on the release of vasoactive molecules by neurons and astrocytes, which act on contractile cells. Vascular smooth muscle cells and pericytes are the contractile cells associated with arterioles and capillaries, respectively, which relax and induce vasodilation. Much progress has been made in understanding the complex signalling pathways of neurovascular coupling, but issues such as the contributions of capillary pericytes and astrocyte calcium signal remain contentious. Study of neurovascular coupling mechanisms is especially important as cerebral blood flow dysregulation is a prominent feature of Alzheimer's disease. In this article we will discuss developments and controversies in the understanding of neurovascular coupling and finish by discussing current knowledge concerning neurovascular uncoupling in Alzheimer's disease.

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Abbreviations: αSMA = alpha smooth muscle actin; AA = arachidonic acid; aECs = arteriolar endothelial cells; APOE4 = apolipoprotein E4; Aβ = amyloid β; BBB = blood–brain barrier; CAA = cerebral amyloid angiopathy; CAMs = capillary-associated microglia; CBF = cerebral blood flow; cECs = capillary endothelial cells; CTTH = capillary transit time heterogeneity; eNOS = endothelial NOS; EP = prostaglandin E2 receptor; ET-1 = endothelin-1; FH = functional hyperaemia; K_{IR} = inward-rectifying potassium channel; mGluR5 = metabolic glutamate receptor 5; NMDA =

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N-methyl D-aspartate; nNOS = neuronal NOS; NOS = nitric oxide synthase; NVC = neurovascular coupling; NVD = neurovascular dysfunction; PGE_2 = prostaglandin E2; ROS = reactive oxygen species; TMPAP = transmembrane prostatic acid phosphatase; tPA = tissue plasminogen activator; VSMC = vascular smooth muscle cell

Introduction

Blood oxygen level-dependent functional magnetic imaging (BOLD fMRI) indicates which regions of the brain are active based on a tight correlation between neuronal activity and increased blood flow to that region, which causes a rapid decrease in paramagnetic deoxyhaemoglobin that can be detected using MRI.[1] This increase in blood flow, termed functional hypaeremia (FH), is underpinned by complex neurovascular coupling (NVC) mechanisms which are not fully understood. Neuronal activity triggers the release of vasoactive agents from neurons and astrocytes.[2] These act on the contractile cells of arterioles and capillaries, which relax and expand vessel diameter, ultimately increasing cerebral blood flow (CBF) locally. Many compounds, including nitric oxide, various arachidonic acid (AA) metabolites, purines and potassium ions, act as vasoactive agents. This review examines the signalling pathways mediated by these compounds.

Building upon earlier reviews, our summary of the field adds updated literature and contextualizes previous experimental evidence of NVC with new evidence of regional heterogeneity of NVC within the rodent brain, which may reconcile existing disagreements in the field regarding molecular mechanisms and the precise microvascular location where NVC is initiated. Discussion of these signalling pathways also primes the discussion of the emerging evidence of neurovascular uncoupling in Alzheimer's disease, which has expanded considerably in the last 5 years.

Vasoactive agents released from within the brain parenchyma can act on cells that reside on the vasculature to elicit a vessel response. Different cell types are located at different levels of the vascular tree. Vascular smooth muscle cells (VSMCs) are the contractile cells associated with arterioles. On capillaries VSMCs are replaced by various subtypes of pericytes. Some studies contest the contribution of capillary pericytes to FH,[3,4] an issue which is addressed in this review.

Understanding the mechanisms of NVC is crucial because neurovascular dysfunction (NVD) contributes to cognitive impairment in Alzheimer's disease[5]—the most prominent cause of dementia, which is expected to double in worldwide prevalence by 2040.[6] Alzheimer's disease is characterized histopathologically by the accumulation of amyloid β (Aβ) into extracellular plaques and the formation of intracellular neurofibrillary tangles consisting of hyperphosphorylated tau protein.[7] NVD plays a large role in Alzheimer's disease pathogenesis, with evidence suggesting that NVD may be the earliest reliable biomarker of Alzheimer's disease.[8] Indeed, NVD may precede and even pave the way for Aβ pathology (supported by the fact that many Alzheimer's disease risk factors are vascular[5]) and Aβ peptides themselves have significant neurovascular effects that are likely to contribute to cognitive decline and disease progression.[9]

Neurovascular coupling in health

Molecular mechanisms of neurovascular coupling

Potential mechanisms for NVC (Fig. 1) have been reviewed extensively.[2,9–12] While there has been a significant increase in our understanding throughout the past two decades, the exact mechanisms at play are still contentious, and we know that there are multiple pathways involved as inhibiting individual mechanisms does not completely abolish NVC. Here we review the current knowledge and add updated literature to the discussion.

Nitric oxide

It is widely accepted that nitric oxide (NO) is a major mediator of NVC.[2,10] Indeed, a systematic review by Hosford and Gourine[13], which analysed data from *in vivo* experiments exploring the effect of pharmacological or genetic knockout of proposed signalling pathways, concluded that blockade of neuronal NO synthase (nNOS) caused the largest reduction in neurovascular response, with an average decrease of 64% across 11 studies. Evidence from mouse cortical slices also suggests that endothelial NO synthase (eNOS) contributes to NVC.[14] Recent work in humans using a non-selective nitric oxide synthase (NOS) inhibitor has confirmed the importance of NO signalling for FH.[15] This work could be repeated in the future using an nNOS-specific inhibitor to test the results of the meta-analysis by Hosford and Gourine.[13]

In neurons, depolarization leads to increases in Ca^{2+} via the opening of voltage-gated calcium channels. This increase in Ca^{2+} activates nNOS in interneurons, leading to the production of the potent vasodilatory agent NO.[16] NO has a direct vasodilatory effect by raising cyclic guanosine monophosphate (cGMP) levels through its receptor; soluble guanylate cyclase (sGC). Lindauer *et al.* found that cGMP application rescues the attenuation of hyperaemic response to whisker stimulation by NOS blockade in rats.[17] However, this study measured changes in regional blood flow but was not specific as to whether application of cGMP was having its effect at the arteriolar or capillary level. When Hall *et al.*[18] used pericyte-specific labelling techniques to isolate the effects of NOS blockade on pericytes and their associated capillaries, they found that NOS blockade inhibited FH, but sGC blockade did not. This suggests that while NO is likely to act to increase cGMP in VSMCs,[17] NO does not act to raise cGMP in pericytes.[18] Hall *et al.*[18] found that when 20-hydroxyeicosatetraenoic acid (20-HETE) is blocked alongside NOS blockade, the previously observed inhibition of FH was lifted. This suggests that in capillary pericytes NO has an indirect vasodilatory effect by inhibiting the production of 20-HETE. 20-HETE is an AA metabolite synthesized via CYP450 and acts by inhibiting large conductance calcium-activated potassium channels (BK_{Ca}), leading to depolarization and vasoconstriction.[19]

Arachidonic acid metabolites

AA is metabolized into several vasoactive agents.[9] Canonically, phospholipase A_2 (PLA_2) was thought to be responsible for the formation of AA,[2] owing to the fact that PLA_2 is expressed in astrocytic endfeet[20] and is thus well placed for a role in FH. However, Mishra *et al.*[21] found no decrease in the hyperaemic

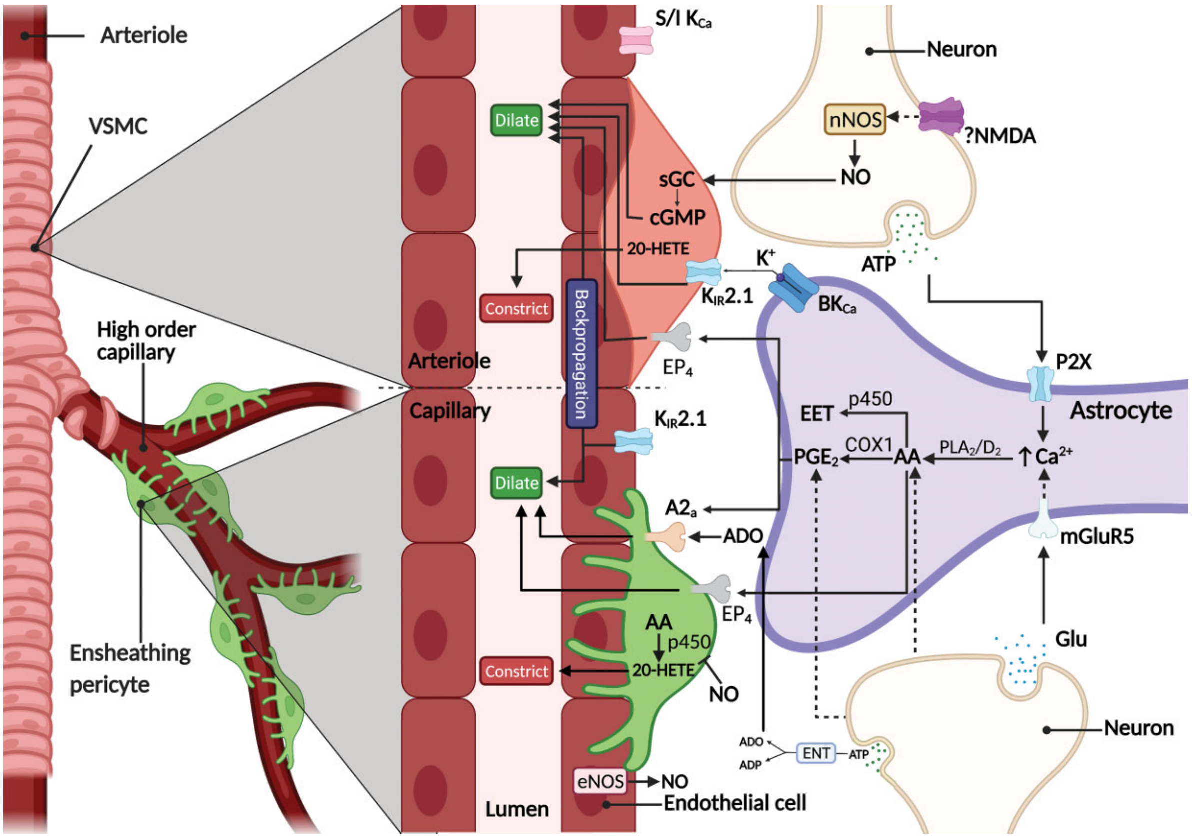

Figure 1 Neurovascular coupling mechanisms covered in this review. The left of the diagram represents a simplification of the cerebrovascular tree. Arterioles branch from pial arteries (not pictured) and are ensheathed in VSMCs. Arterioles in turn branch into capillaries, which are associated with pericytes. The right of the diagram represents the neurovascular unit (composed of mural cells, endothelial cells, neurons and glia) and the NVC mechanisms described in this section. Dashed lines represent pathways with limited or debated evidence. Created with BioRender.com. 20-HETE = 20-hydroxyeicosatetraenoic acid; BK_{Ca} = large conductance calcium-activated potassium channel; cGMP = cyclic guanosine monophosphate; COX1 = cyclooxygenase 1; EK = equilibrium potential of potassium; PLA_2 = phospholipase A2; PLD_2 = phospholipase D2; S/IK_{Ca} = small and intermediate conductance calcium-activated potassium channel; sGC = soluble guanylate cyclase.

response to neuronal activity when PLA_2 was pharmacologically blocked. Upon further investigation, they were able to identify the phospholipase D2 (PLD_2) isoform as the initiator of AA synthesis.[21] The vasodilatory species prostaglandin E_2 (PGE_2) and various epoxyeicosatrienoic acids (EETs) are synthesized from AA by cyclooxygenase 1 (COX1) and p450 enzymes, respectively.[2]

Two studies by Attwell's laboratory have isolated the receptor through which PGE_2 has its effects on capillary pericytes as the prostaglandin E2 receptor 4 (EP_4) receptor.[18,21] EETs have been shown to contribute to NVC at the capillary level and are also thought to act at the EP_4 receptor.[22] The EP_4 receptor is a G_s linked G-protein-coupled receptor (GPCR), thus its activation increases intracellular cAMP, which phosphorylates myosin light chain kinase and leads to vasodilation.[23] The role of the EP_4 receptor has been extended to arteriolar VSMCs by Czigler *et al.*,[24] who found that EP_4 blockade inhibits the dilation of isolated human parenchymal arterioles. Furthermore, at higher concentrations, PGE_2 application caused vasoconstriction rather than the vasodilation observed in low concentrations. As this constriction is blocked by an EP_1 receptor antagonist, Czigler *et al.*[24] conclude that PGE_2 acts biphasically via EP_4 and EP_1 receptors at low and high concentrations, respectively. The EP_1 receptor is a GPCR that mainly signals via G_q, thus activation increases $[Ca^{2+}]_i$, leading to vasoconstriction.[25]

Mishra *et al.*[21] report that astrocyte Ca^{2+} is not involved in arteriolar dilation. As the release of AA metabolites seemingly relies on an astrocytic Ca^{2+} signal,[2] this could imply that AA metabolites do not act on arteriolar VSMCs. Nevertheless, in a review by Nippert *et al.*,[26] the authors hypothesize a mechanism that bypasses this apparent problem. Nippert *et al.*[26] propose that neurons release AA and PGE_2. Thus AA can diffuse into astrocytes, which can synthesize and release EETs without an astrocytic Ca^{2+} signal. Although this hypothesis is supported by expression of PLA_2 and PLD_2 in neurons, giving a feasible pathway for neuronal AA release,[27] experimental confirmation is needed.

Purinergic signals

Astrocyte Ca^{2+} was nonetheless shown to be necessary for the dilation of capillaries via pericytes, but not arteriolar dilation by VSMCs, in the rat cortex.[21] Similarly, Ca^{2+} signals in Müller cells, a specialized type of astrocyte in the retina, were shown to be vital for retinal capillary dilation.[28] But how is this astrocyte Ca^{2+} signal initiated?

Previously, it has been suggested that astrocytic metabotropic glutamate receptor 5 (mGluR5) activation by glutamate released at the synapse is responsible for this calcium signal.[2] However, several lines of evidence make the role of astrocytic mGluR5 dubious. First, while mGluR5 may play a role in the juvenile brain, it was found to be downregulated in the astrocytes of adult mice[29] and rats.[30] Second, despite achieving an 80% blockade of mGluR5 *in vivo*, Calcinaghi *et al*.[31] did not find the diminished hyperaemic response predicted if mGluR5 is key. Third, inositol 1,4,5-trisphosphate receptor type 2 (IP3R2), which mediates the mGluR5–GPCR signalling pathway that ends in Ca^{2+} release from intracellular stores, may not be needed for an intact hyperaemic response. This was shown *in vivo* by two studies using IP3R2-knockout (KO) mice that had an intact hyperaemic response in both the visual[32] and somatosensory cortex.[33] It should be mentioned that glutamate may also act at endothelial *N*-methyl D-aspartate (NMDA) receptors, knockout of which has been shown to reduce FH mediated by penetrating arterioles, precapillary arterioles and capillaries despite having no effect on whisker stimulation-evoked neuronal Ca^{2+} signals in the mouse somatosensory cortex.[34]

With the role of mGluR5 in doubt (and indeed found not to affect capillary dilation *in vitro*), Mishra *et al*.[21] instead suggest that the P2X receptor mediates this astrocyte $[Ca^{2+}]_i$ signal. As opposed to the P2Y receptor, which has previously been suggested to have a role in NVC, but being reliant on IP3 generation is an unlikely candidate for the same reason as mGluR5,[32,33] the P2X receptor is a ligand-gated ion channel activated by ATP and permeable to Ca^{2+} (as well as other cations).[35] Supporting evidence comes from the pharmacological blockade of these P2X receptors, which significantly attenuates stimulation-evoked capillary dilation.[21]

In addition to its action at the P2X receptor, ATP is also rapidly metabolized by ectonucleotidases (ENT) into both ADP and adenosine, which are likely to be active in NVC.[2] Dirnagl *et al*.[36] found that local application of theophylline, a non-selective adenosine receptor antagonist, led to reductions in stimulation-evoked regional CBF increases *in vivo*. This study used laser Doppler flowmetry to measure CBF changes, thus while it confirmed a role for adenosine in FH, it did not show whether this was mediated by capillary pericytes or arteriolar VSMCs. On the other hand, Ko *et al*.[37] used a video micrometer to show that theophylline attenuates pial arteriole dilation following neuronal stimulation *in vivo*. Additionally, this group also found that the adenosine reuptake inhibitors dipyridamole and inosine, which would increase extracellular adenosine levels, increased pial arteriole dilation in response to stimulation.[37] Together these results strongly suggest that adenosine released by neuronal activity causes pial arteriolar dilation.[37] Matsugi *et al*.[38] found that pericytes can also relax in response to adenosine *in vitro*. Application of adenosine onto cultured bovine pericytes invoked pericyte relaxation, which was not reversed by a selective A_1 receptor antagonist, but was reversed by a selective A_2 receptor antagonist, suggesting that this adenosine-invoked pericyte relaxation is mediated by the A_2 receptor.[38] This finding has been confirmed in cultured human pericytes, which relax *in vitro* via an A_1/A_{2a} receptor-dependent mechanism.[39]

The vasodilatory role of adenosine in FH is further supported by work using microelectrode fast scan cyclic voltammetry,[40] a technique useful due to its ability to simultaneously measure adenosine and oxygen transients.[41] Wang and Venton[40] suggest that, as these oxygen transients are highly likely to be a result of increased regional blood flow, they represent the vasodilation caused by neuronal activity. The study not only showed correlation between adenosine and oxygen transients, but also that the adenosine transient preceded the oxygen transient by 0.2 s.[40] These findings taken together strongly imply that adenosine acts as a vasodilatory agent in FH. Furthermore, the same group tested A_1 and A_{2a} antagonists to establish which receptor mediated this effect, finding that, while A_1 blockade had no effect, A_{2a} blockade caused a 32% diminished oxygen transient.[40] The A_{2a} receptor is expressed on VSMCs and is a G_s-linked GPCR, meaning that activation of the A_{2a} receptor by adenosine leads to increased cAMP, VSMC relaxation and vasodilation.[42] It should be noted that the involvement of A_1 and A_{2a} receptors is complicated by the fact that both receptors are expressed neuronally, where they can play a (mostly) inhibitory and excitatory role, respectively.[43]

Wells *et al*.[44] used a lentiviral vector to overexpress transmembrane prostatic acid phosphatase (TMPAP) in the somatosensory cortex of rats. TMPAP is a potent ENT, thus overexpression promotes the rapid metabolism of ATP into adenosine and ADP.[44] They found that TMPAP rats had a significantly lower BOLD fMRI signal compared to the controls.[44] BOLD fMRI relies on CBF increases generated by NVC,[1] thus this diminished signal suggests that TMPAP overexpression attenuates NVC. As adenosine is a product of TMPAP-catalysed dephosphorylation of ATP, TMPAP overexpression has the dual effect of increasing extracellular adenosine alongside decreasing the actions of ATP released from neurons. Therefore, these results may suggest that the actions of ATP at the P2X receptor are more important for FH than the action of adenosine at the A2a receptor.

Potassium ions

Inward-rectifying potassium (K_{IR}) channels are expressed on VSMCs and are sensitive to increases in extracellular potassium ($[K^+]_o$).[45] K_{IR} receptor activity in response to increased $[K^+]_o$ leads to hyperpolarization of the cell.[46] This is because K_{IR} channel conductance has an unusual dependence on $[K^+]_o$.[47] A rise in $[K^+]_o$ simultaneously shifts the value of the K^+ equilibrium potential (E_K) positive (i.e. outward current through K_{IR} channels decreases), but also increases channel conductance. This latter effect dominates, thus a rise in $[K^+]_o$ increases the outward K^+ current above E_K and causes VSMC hyperpolarization and relaxation.

K_{IR} channels are also expressed on capillary endothelial cells (cECs). Hyperpolarization of cECs in response to rises in $[K^+]_o$ can rapidly backpropagate along neighbouring cECs to upstream arterioles, thus initiating a retrograde local vasodilatory response. The strong inward-rectifying $K_{IR}2.1$ channel has especially been implicated, with Longden *et al*.[45] developing an endothelial cell-specific $K_{IR}2.1$ knockout mouse model and finding that CBF increases in response to whisker stimulation were reduced by 50% in comparison to wild-type mice. These findings imply a key role for cECs in sensing increases of $[K^+]_o$ and propagating a hyperpolarizing signal to upstream arterioles, which modelling studies support.[48]

A much-cited source of this increase in $[K^+]_o$ is an increase in Ca^{2+} in astrocyte endfeet, which activates BK_{Ca}, inducing an outward K^+ current.[49] In support of this pathway, Girouard *et al*.[50] found that the selective BK_{Ca} channel antagonist paxilline significantly inhibited FH *in vitro*. It is unclear how these results relate to more recent *in vivo* evidence against the role of astrocytic Ca^{2+} in arteriolar dilation.[21] A possible explanation for these contrasting results is the difference in experimental methods. While Girouard *et al*.[50] utilized electrical field stimulation as an *in vitro* model of neuronal activation, Mishra *et al*.[21] were able to use forepaw stimulation *in vivo*. Brain slice study of NVC may be problematic due to the release of vasoactive compounds during excision of brain tissue, as well as the need to pharmacologically preconstrict blood vessels due to a lack of vascular tone.[13,51] Indeed, following the results of their systematic review, Hosford and Gourine[13] found that, while *in vivo* results generally agree with other studies in the field, *in* *vitro* results show greater discordance. Nevertheless, an increase in $[K^+]_o$ could result from other sources such as activation of BK_{Ca} channels by EETs[52] or K^+ efflux via potassium channels during neuronal repolarization.[45] Furthermore, small and intermediate conductance K_{Ca} (S/IK_{Ca}) channels are expressed on endothelial cells and cause pressurized arterioles to constrict when they are blocked in brain slice experiments and reduce resting CBF when blocked *in vivo* in mice.[53] Future study should elucidate the contribution of these channels to FH.

Recent work has provided evidence for another trigger of the backpropagating K_{IR}-induced hyperpolarization. Thakore *et al.*[54] found that transient receptor potential ankyrin 1 (TRPA1) channels on cECs [thought to be activated by reactive oxygen species (ROS) released from active neurons and astrocytes] initiate a Ca^{2+} signal that triggers a rapidly backpropagating K_{IR} current leading to dilation of upstream arterioles. This Ca^{2+} signal is propagated by a pannexin-1/P2X receptor dependent pathway and eventually leads to activation of small/intermediate conductance K^+ channels, raising $[K^+]_o$ and triggering K_{IR} channels.[54] The authors show the significance of this mechanism for FH using genetic knockout and pharmacological inhibition studies, which both show reduced functional hyperaemic response to whisker stimulation of 5 s (but not 1 or 2 s, suggesting other mechanisms may have accounted for the more transient responses).

Metabolic signalling

Metabolic feedback mechanisms propose that the detection of decreased O_2/glucose, or the build-up of metabolic waste products (CO_2/lactate) resulting from increased neuronal metabolism, could drive the haemodynamic response to active regions of the brain.[2] It should be noted that the hyperaemic response exceeds the increase in cerebral metabolism by ∼6-fold, although it is possible that the brief period of increased demand that precedes the CBF increase is sufficient for transient changes in metabolite levels to evoke this change.[55]

It has been shown that the depletion of glucose due to neuronal activity is unlikely to act as a signal in FH. Powers *et al.*[56] found that regional CBF did not increase in response to progressive hypoglycaemia. Furthermore, hyperglycaemia did not hinder the haemodynamic response to somatosensory stimuli in the rat cortex.[57] Similarly, this group reported that hyperoxia has no effect. This is in line with more recent work showing that hyperbaric hyperoxygenation, which maintains haemoglobin oxygen saturation, has no effect on the haemodynamic response to neuronal stimulation.[58] While Wei *et al.*[4] repeated this finding, they also showed that hyperbaric hyperoxia does not eliminate the transient dips in pO_2 that follow neuronal excitation, which they propose to be the key signal rather than the basal O_2 levels tested in previous studies. In contrast, sodium cyanide (NaCN) inhibits oxidative phosphorylation and thus does eliminate transient dips in pO_2. Upon careful selection of a dose that did not affect neuronal excitation, it was shown that NaCN reduced capillary hyperaemia by up to 78% *in vivo*. This capillary hyperaemia preceded arteriolar hyperaemia and is a result of improved erythrocyte deformability in response to pO_2 dips, which facilitates increased flow through the narrow capillary lumen.[4] However, inhibition of oxidative phosphorylation using NaCN is likely to have many unpredictable effects, including increased levels of adenosine,[59] which may confound Wei and colleagues' results.

Microinjection of O_2 scavengers such as sulphite induce a rapid local pO_2 dip, and Wei *et al.*[4] showed that this microinjection causes a capillary hyperaemia that is not affected by pharmacological blockade of K_{IR} channels (using Ba^{2+}), NOS, PGE_2 production (using indomethacin, a non-selective COX inhibitor) or A_{2A} receptors. This indicates that the capillary hyperaemia induced by transient dips in O_2 is independent of the major vasoactive agents shown in Fig. 1. Wei *et al.*[4] propose that this hyperaemic response is instead the result of increased erythrocyte deformability, which increases flow velocity. The finding that inhibition of the major NVC mechanisms does not affect capillary hyperaemia induced by sulphite microinjection contrasts with many studies that report attenuated capillary hyperaemia following inhibition of these pathways.[13,18,38] This may be explained by the fact that decreases in pO_2 due to microinjections of sulphite are not analogous to physiologically relevant transient dips in pO_2 caused by neuronal activity.

In summary, there are a myriad of pathways and cell types contributing to the mechanisms of NVC and the tight control of CBF locally. This may indicate a level of redundancy, allowing FH to continue should another pathway become deficient. Hosford and Gourine[13] estimate that ∼40% of the neurovascular response remains unaccounted for after considering the feedforward mechanisms described above, dependent on neurotransmitter and potassium release from neuronal activity. Our knowledge of NVC is evidently still incomplete; further research could focus on the perhaps underappreciated roles of metabolic[13] or cEC[45,48] mediated mechanisms. Furthermore, although the role of cECs has been explored, the role of arteriolar endothelial cells (aECs) in signalling is not well characterized. Caveolae in aECs have been shown to be important for NVC beyond their known role as mediators of eNOS signalling.[60] Chow *et al.*[60] found that knocking out caveolae either genetically or by suppressing expression ectopically, arteriolar dilation in response to whisker stimulation was decreased despite no change in resting CBF or neural activity. aEC KO had an additive effect to eNOS KO, suggesting that aECs signal independently of the eNOS pathway. The authors hypothesize that caveolae of aECs mediate signalling by clustering receptors,[60] although further work is needed to elucidate which receptors/signalling pathways are involved and how aEC caveolae are affected in disease.

Other cells outside of the classical neurovascular unit may also influence NVC. Recently, capillary-associated microglia (CAMs) have been shown to exert control on CBF.[61] Pharmacological knockout of CAMs caused a generalized increase in CBF but a reduced CBF response to CO_2 stimulus.[61] The authors argue that given $P2Y_{12}$ or pannexin 1 (PANX1) genetic KO produced similar effects, the vascular influence of CAMs is dependent on this pathway.[61] We would argue that this is insufficient evidence to make this claim. First, the $P2Y_{12}$ receptor is expressed elsewhere, including VSMCs,[62] and so their KO is likely to have effects beyond CAMs. Second, an experiment combining CAM knockout with $P2Y_{12}$/PANX1 showing no or less than expected additive effect is needed to show overlap of $P2Y_{12}$/PANX1 and CAM neurovascular regulation. Nevertheless, future study could focus on the effects CAM knockout on neuronal stimulation-evoked FH.

Do pericytes constrict and dilate capillaries?

Despite extensive research into the signalling pathways underlying NVC, there remains debate as to whether capillaries can actively dilate to contribute to FH rather than just passively dilating due to increased flow in arterioles.[63]

Along the vascular bed, pericytes can be broken down into subtypes based on their morphology and alpha smooth muscle actin (αSMA) expression.[63,64] In a recent review by Hartmann and colleagues,[65] the authors use a system of nomenclature that matches each microvascular zone to its corresponding mural cell, building on work by Grant *et al*.[64] to classify pericytes using αSMA expression and morphology. Penetrating arterioles are covered by smooth muscle cells that form continuous rings. The arteriole–capillary transition (ACT) zone is covered by ensheathing pericytes. The ACT zone has previously been a source of confusion, given that the zone itself has been termed the precapillary arteriole[63] and transitional zone,[66] and the mural cells here have been termed junctional pericytes[67] or transitional pericytes. These cells, termed ensheathing pericytes by Grant *et al*.,[64] have ovoid cell bodies which classify them as pericytes. This forms the rationale behind the switch from precapillary arteriole to ACT zone to avoid the confusion of a pericyte being associated with an arteriole.[65] Precapillary sphincters have also been identified at the junction of the penetrating arteriole and ACT.[68] Two pieces of experimental evidence support the active dilation of this cell type: *ex vivo* detection of αSMA expression and *in vivo* images showing dilation in response to whisker stimulation.[68] Recent evidence suggests that these precapillary sphincters, as well as first-order capillaries, are more highly responsive to a range of stimuli than penetrating arterioles or capillary pericytes.[69] Using *in vivo* 4D two-photon microscopy, Zambach and colleagues[69] showed that diameter changes in response to whisker pad stimulation, acetylcholine, endothelin-1 (ET-1) and other stimuli occurred at similar kinetics from penetrating arterioles to downstream capillaries; however, the magnitude of response was greatest in precapillary sphincters and first-order capillaries, and modelling suggested that sphincters had the greatest impact on flow through the microvascular network. Lower-order capillaries (branches 1–4) are covered by mesh pericytes, whereas higher-order capillaries (branches 5 and above) are covered by thin strand pericytes.[64] These latter two subtypes are termed capillary pericytes by some.[67]

There is significant evidence that pericytes actively contribute to FH. Hall *et al*.[18] found that when *in vivo* sensory stimuli increased CBF, capillaries were responsible for 84% of this rise and that capillary dilation preceded arteriolar dilation. Mishra *et al*.[21] found that arteriolar and capillary dilation were mediated by distinct signalling pathways, suggesting that capillaries dilate actively rather than passively as a result of arteriolar hyperaemia. Furthermore, Kisler *et al*.[70] reported decreased global haemodynamic response to stimulation in pericyte-deficient mice, which was attributed to significantly delayed capillary dilation.

However, other studies have found that pericytes do not express the contractile protein αSMA and thus conclude that capillary pericytes cannot actively relax.[3,4] Fundamentally, this discrepancy appears to be based on a disagreement in pericyte definition, which becomes ambiguous towards the arteriolar junction.[63] It has also been suggested that post-mortem detection of αSMA in pericytes may be difficult due to rapid actin depolymerization.[66] This group found that when snap freeze fixation of actin with methanol is used, αSMA is detectable in pericytes and ensheathing pericytes express more αSMA than pericytes distal to the arteriolar junction. This supports the proposition that pericytes closer to the arterioles are more involved in the active dilation of capillaries because they express more αSMA and have more circumferential processes.[63] Indeed, in the study by Zambach *et al*.[69] αSMA expression mirrored mural cell responsiveness and pressure distributions in their network models, with precapillary sphincters having the highest expression.

Capillary pericytes (mesh and thin strand pericytes) express less αSMA and largely have longitudinal rather than circumferential processes. Grutzendler and Nedergaard[51], who define pericytes as homogenous noncontractile mural cells, take these cells to be the only 'true' pericytes and claim that they do not express any αSMA. This is disputed by some cell culture evidence showing that pericytes are αSMA-positive, and that there are distinct populations of pericytes that are heterogenous for αSMA expression.[71]

The network of capillary pericytes is complicated. Pericyte projections may wrap around several capillaries, and there is evidence that these projections can act independently to exert differential control on different capillaries.[72] Furthermore, interpericyte tunnelling nanotubes (IP-TNTs) connect pericytes in the mouse retina and allow for intercellular conduction of Ca^{2+} waves and communication between pericytes to facilitate NVC.[73]

Transcriptome analysis of capillary pericytes has revealed myosin expression but absent or very low expression of *Acta2* (αSMA).[74] Nevertheless, a recent study has shown that following optogenetic stimulation, pericytes exert a slow vasoconstriction that slows red blood cell flux.[75] This optogenetically induced vasoconstriction is abolished by inhibiting Rho-kinase, a key mediator of contraction in smooth muscle cells,[76] providing strong evidence for capillary pericyte contraction via this cellular contractile mechanism rather than other influences.[75] The authors suggest that the slow control of capillary pericytes may reflect the low expression of *Acta2* or indicate other slower contractile mechanisms at play such as those involving the cytoskeleton.[75] It is possible that higher-order capillaries rely on atypical contractile mechanisms, such as *de novo* polymerization of filamentous actin, allowing for contraction despite low levels of αSMA.[77,78]

With differing criteria for the identification of pericytes, it is inevitable that groups will report different conclusions concerning pericyte-mediated capillary dilation.[3,63] Reaching a consensus on a robust methodology of labelling pericytes may aid in solving this ambiguity. Platelet-derived growth factor receptor beta (PDGFRβ) and neural/glial antigen 2 (NG2) markers are widely used to identify pericytes, but their selectivity for pericytes over VSMCs is unclear and requires the use of morphological criteria and cell position along the vascular tree.[51,79] Recently, a labelling technique using the fluorescent dye NeuroTrace 500/525 has demonstrated complete selectivity for pericytes.[80] However, while the selectivity of this dye will certainly be useful in future studies, the authors began by defining pericytes as noncontractile, thus NeuroTrace 500/525 alone cannot settle the debate as to whether pericytes actively contribute to vasomotility. Furthermore, NeuroTrace does not label αSMA[80] and thus will not label ensheathing pericytes that are αSMA-positive. However, NeuroTrace selectivity for capillary pericytes has been verified by another group.[64]

Despite this, an overwhelming amount of imaging and labelling evidence now supports the contractile properties of pericytes most proximal to the arteriolar junction, which allow them to tightly regulate CBF through control of capillary diameter.[66,72,75,81,82]

Evidence of region and layer heterogeneity

Although there has been evidence suggesting regional heterogeneity in neurovascular coupling for over 20 years,[83] recent advances have given us mechanistic insight into regional as well as laminar regulation of CBF. In 2018, Rungta *et al*.[84] found that in the olfactory bulb, odour stimuli cause a rapid Ca^{2+} transient across an entire vascular arbour but that the parenchymal arteriole and first-order capillary trigger FH by dilating first, followed by higher-order capillaries as well as the pial artery. Although the greatest increase in red blood cell velocity was found in higher-order capillaries, the thin-stranded pericytes associated with these capillaries were not responsible for triggering FH, given that the Ca^{2+} transients in these pericytes did not precede the increase in red blood cell velocity.[84] This led the authors to hypothesize that the delayed response of these thin-strand pericytes may facilitate rather than trigger FH. The authors suggest that the fact the pericytes closer to the synapse are closer to any vasoactive signalling, but paradoxically dilate later, may reflect weaker coupling to the endothelial back propagating K^+ current. This is in line with the finding that pericyte vasomotility is slow but nonetheless necessary for effective FH.[75] More recently, Rungta *et al.*[65] studied FH in the somatosensory cortex in response to whisker stimulation and found that heterogeneity between arbours is much higher in this brain region, in which no clear microvascular compartment (pial artery, parenchymal arteriole, first-order capillary or higher-order capillaries) dilated earliest across arbours. This is contrary to Hall *et al.*,[18] who reported that both first-order and higher-order capillaries dilated earlier than arterioles in the somatosensory cortex. Methodological differences including anaesthesia procedures, which have been shown to delay NVC,[65] may in part explain these differences.

Shaw *et al.*[85] found that, compared to the visual cortex, the CA1 subfield of the hippocampus has a weaker capacity for NVC as well as overall lower oxygenation. The CA1 subfield exhibits preferential neuronal loss in Alzheimer's disease[86] as well as pericyte injury-associated blood–brain barrier (BBB) breakdown in mild cognitive impairment[87] and is therefore of particular interest. Compared to the visual cortex, neuronal calcium peaks (which are analogous to neuronal activity) triggered fewer and weaker vasodilation in the hippocampus, even though calcium peaks were on average large in the hippocampus.[85] *In vivo* study of the mouse hippocampus, for which this paper is novel, is crucial, given the susceptibility of this region to hypoxia and Alzheimer's disease. Due to the lack of a simple afferent for the hippocampus (c.f. whisker stimulation for the somatosensory cortex, or odour for the olfactory bulb), this was achieved by temporal and spatial matching of neuronal calcium spikes with vasodilation to show FH. The authors' model shows that the lower oxygenation of the hippocampus makes oxygen delivery a limiting factor for ATP synthesis, which would only be exacerbated by weaker FH. By extension, it is reasonable to assume the hippocampus would also be more susceptible to any pathological NVC deficits (such as neurovascular uncoupling in Alzheimer's disease, see latter sections). This may explain why the hippocampus is particularly vulnerable to hypoxia as well as Alzheimer's disease pathogenesis.[85,88]

The reasons underlying these regional differences are being investigated. Rungta *et al.*[84] hypothesize that the difference between the olfactory bulb and the somatosensory cortex may be due to a higher synaptic concentration in the olfactory bulb, leading to less heterogeneity between arbours and a clear temporal pattern of vasomotion. Analysing differences between mRNA expression between cells in the visual cortex *versus* the hippocampus, Shaw *et al.*[85] found no differences in capacity to produce vasoactive mediators in neurons or astrocytes between both regions (including NO, EETs). However, the study did find lower mRNA expression of proteins in mural cells (myosin light chain phosphatase) as well as endothelial cells ($K_{IR}2.1$, a NO receptor subunit and PGE synthase), which would predispose the hippocampus to weaker NVC.

Modelling experiments demonstrate variability between cortical layers in terms of which vessel type contains the highest pressure drop experienced by traversing red blood cells, which the authors argue is a crucial determinant of flow regulation.[89] A previous study found that the greatest vascular resistance is contained within capillaries proximal to the penetrating arteriole (although the type of capillary was not verified by staining, only by branch order and vessel diameter), leading the authors to argue capillary dilation would have the greatest influence on NVC.[90] Schmid and colleague's findings agree that for superficial cortical layers the pressure drop in capillaries is largest; they found that this is not true for deeper cortical layers, in which arterioles contain the largest pressure drop and are thus more likely to be the site of NVC initiation.[89]

Regional differences in NVC, particularly comparisons between regions with varying vulnerability to Alzheimer's disease pathology, are a rich field for further study. Further *in vivo* animal research studying differences in NVC between vulnerable and resistant regions/layers such as the recent study comparing CA1 (vulnerable) and the primary visual cortex (resistant) by Shaw and colleagues[85] are warranted. These studies could look at control animals (in which NVC differences suggest a baseline characteristic that may underlie vulnerability to Alzheimer's disease pathology) or animal models of Alzheimer's disease. Building upon Shaw and colleagues' work,[85] comparison could be made between regions with differing vulnerability to Alzheimer's disease within the cerebral cortex itself, for example the primary visual cortex and the relatively more vulnerable visual association cortex.[91] Post-mortem studies on human tissue could look at differences in the neurovascular unit between regions. For example, capillary vasoconstriction[92] has been measured as a neurovascular change in human Alzheimer's disease biopsy, and studies could compare the extent of vasoconstriction across regions resistant and vulnerable to Alzheimer's disease pathology.

Layer-specific changes in Alzheimer's disease should also be studied. Two-photon microscopy data in mice show layer-specific differences in microvascular haemodynamics throughout cortical layers, with layer IV exhibiting the least capillary transit time heterogeneity (CTTH) and thus achieving greatest oxygen delivery.[93] High-resolution fMRI data in anaesthetized macaques also shows differences in NVC between cortical layers.[94] fMRI has also shown that CTTH is elevated in patients with Alzheimer's disease[95,96] and mild cognitive impairment.[97] Thus, any relationship between CTTH and selective vulnerability to Alzheimer's disease pathology (for instance, plaque pathology preferentially effects cortical layers II and III, while tangles predominate in V[91]) should be investigated on a layer-specific basis. Pericyte coverage differs between cortical layers in mice,[98] but human data are needed, particularly studies of layer-specific neurovascular unit changes in post-mortem Alzheimer's disease human tissue.

A further intriguing yet poorly understood factor is how some with Alzheimer's disease neuropathology remain asymptomatic (reviewed elsewhere[99]). Neurovascular differences between this population and Alzheimer's disease patients have not been studied but may reveal compensatory mechanisms that elucidate disease processes and treatment targets.

Neurovascular uncoupling

The two-hit vascular hypothesis for Alzheimer's disease progression proposes that NVD promotes Aβ pathology, and Aβ peptides in turn have deleterious effects on the cerebral vasculature (forming a positive feedback loop).[5] In support of this hypothesis, in the Alzheimer's Disease Neuroimaging Initiative Iturria-Medina *et al.*[8] found that vascular dysfunction was the most prominent and potentially earliest abnormality in Alzheimer's disease. Indeed, genetic Alzheimer's disease risk factors such as apolipoprotein E4 (*APOE4*) have been independently associated with direct vascular effects, further supporting NVD as an early event in Alzheimer's disease.[100] NVD in Alzheimer's disease includes BBB dysfunction, CBF reductions, cerebral amyloid angiopathy (CAA) and neurovascular uncoupling.[10] Recently, new evidence has emerged that supports the role of vascular dysfunction in Alzheimer's disease. The evidence of intrinsic differences between the vascular networks of the hippocampus and the cortex may provide the link between NVD and the selective regional vulnerability observed in Alzheimer's disease (with the hippocampus being most vulnerable).[85]

It is widely reported that CBF reductions are found in the Alzheimer's disease brain and that these reductions contribute to cognitive decline.[101,102] Although it is likely that the lower metabolic demand of an atrophied brain results in lower CBF,[103] evidence that CBF reductions precede significant neurodegeneration[104] suggests that other factors contribute to cerebral hypoperfusion in Alzheimer's disease. In addition to global hypoperfusion, there is also evidence of neurovascular uncoupling (an impairment of the physiological mechanisms linking neuronal activity to a regional CBF increase) in both Alzheimer's disease patients[105] and mouse models of Alzheimer's disease.[106,107] This neurovascular uncoupling likely contributes to cognitive impairment as well as paving the way for 'hit two' of the two-hit vascular hypothesis by potentiating the effects of Aβ.[5,9,108] This is emphasized by studies in mice showing improvements in cognitive function in Alzheimer's disease mice once NVC is restored.[106,109]

Understanding NVC in dementia is also crucial to our ability to interpret functional imaging, which has been widely used to probe the neural processing changes in cognitive impairment and has potential use in prognostication and early detection. For instance, *APOE4* carriers have been reported to have increased amplitude and regional extent of activation in fMRI during memory tasks in the absence of diagnosed cognitive impairment.[110] Others have found that high-risk subjects (based on *APOE4* carrier status and family history) have decreased inferotemporal activation during visual naming and letter fluency tasks.[111] Resting state connectivity is also altered by *APOE4* even in the absence of detectable neuropathology or biomarkers.[112] In Alzheimer's disease itself, there are numerous reported observations on dysfunction of default-mode/resting state networks,[113,114] memory networks[115] and other fMRI abnormalities. However, some findings have been criticized due to non-specificity for Alzheimer's disease risk genes[116] as well as their failure to take into account potential vascular changes—such as altered NVC, increased baseline perfusion and decreased fractional responsiveness to a task, despite no differences in overall perfusion.[117] As such, mechanistic studies of NVC in Alzheimer's disease are essential to determine whether the differences seen on functional imaging reflect altered neural activity or underlying vascular changes.

This section reviews the current knowledge of the mechanisms underlying neurovascular uncoupling in Alzheimer's disease (represented in Fig. 2 and summarized in Table 1) by making a conceptual distinction between disrupted signalling and physical mechanisms (such as reduced vasomotility or occlusion); however, in reality, these are of course highly interlinked issues. It is important to note that much of our understanding is based on transgenic animal models of Alzheimer's disease, typically featuring very high levels of Aβ expression to replicate the pathology in shorter lifespan rodents. The lack of success with anti-Aβ therapies in clinical trials has recently called the amyloid hypothesis into question; however, it remains our best unifying theory of Alzheimer's disease, and while the complex interplay between Aβ, tau and other pathologies needs to be elucidated further (reviewed elsewhere[118]), these models remain our best tool to investigate NVC in Alzheimer's disease.

Aberrant signalling pathways

Potassium dysfunction

As discussed earlier, $K_{IR}2.1$ channels contribute to NVC by facilitating a retrograde vasodilatory signal from cECs to upstream arterioles.[45] In a recent study by Mughal *et al.*,[119] it was shown that this $K_{IR}2.1$ current is deficient in mouse models of Alzheimer's disease. Furthermore, the finding that blockade of $K_{IR}2.1$ using Ba^{2+} diminished hyperaemic responses in control mice but not Alzheimer's disease mice indicates that the latter have already attenuated $K_{IR}2.1$ activity.[119] PIP2 is a phospholipid that is key for the activation of $K_{IR}2.1$ channels.[120] Mughal *et al.*[119] found that administration of a PIP2 analogue not only restored the cEC $K_{IR}2.1$ current but also increased the hyperaemic response to whisker stimulation in Alzheimer's disease mice. This latter experiment was not repeated in control mice. Although the authors showed that PIP2 administration has no effect on the $K_{IR}2.1$ current of control mice cECs *in vitro*, an *in vivo* experiment testing the effects of PIP2 on FH in control mice would buttress their evidence of PIP2-mediated $K_{IR}2.1$ signalling deficiency in Alzheimer's disease mice. The same group noted similar effects in a mouse model of vascular dementia (CADASIL), suggesting PIP2 deficiency-related $K_{IR}2.1$ inactivity may be a shared feature across dementia subtypes.[121]

Nitric oxide dysfunction

Aberrant NO signalling has been implicated in Alzheimer's disease for decades.[122] As discussed above, the NMDA–NOS–NO pathway is particularly crucial to NVC, and there is evidence of dysfunction in each step of the pathway in Alzheimer's disease and ageing.

Tissue plasminogen activator (tPA), beyond its fibrinolytic functions, also influences NVC and has been shown to be deficient in Alzheimer's disease via upregulation of plasminogen inhibitor-1 (PAI-1).[106,123] tPA-deficient mice have a reduced functional hyperaemic response to whisker stimulation compared with control mice.[124] This effect was shown to be attributable to reduced NMDA-dependent phosphorylation of nNOS, which likely attenuates NO release and NVC.[124] More recently, the same group has shown that tPA activity is deficient in Alzheimer's disease mice and that these mice exhibit a reduced functional hyperaemic response to whisker stimulation reversed upon exogenous tPA application, an effect dependent on NMDA-induced NO release.[106] The functional hyperaemic response in Alzheimer's disease mice is also restored by PAI-1 inhibition, which was associated with improved performance by Alzheimer's disease mice in tests of cognitive function.[106] Of note, $A\beta_{1-40}$ application, which has been shown to attenuate FH,[125] reduces the response to whisker stimulation in wild-type mice but not in PAI-1-deficient mice.[106] These results link Alzheimer's disease-related PAI-1 upregulation/tPA deficiency, $A\beta_{1-40}$ and the dampening of the NMDA–nNOS–NO pathway. Given that tPA also has effects on other determinants of cognitive function, such as synaptic plasticity,[126] Park *et al.*[106] suggest that tPA/PAI-1 could be an important target of therapy.

Efforts have been made to study the expression of the three isoforms of NOS, endothelial (eNOS), neuronal (nNOS) and inducible

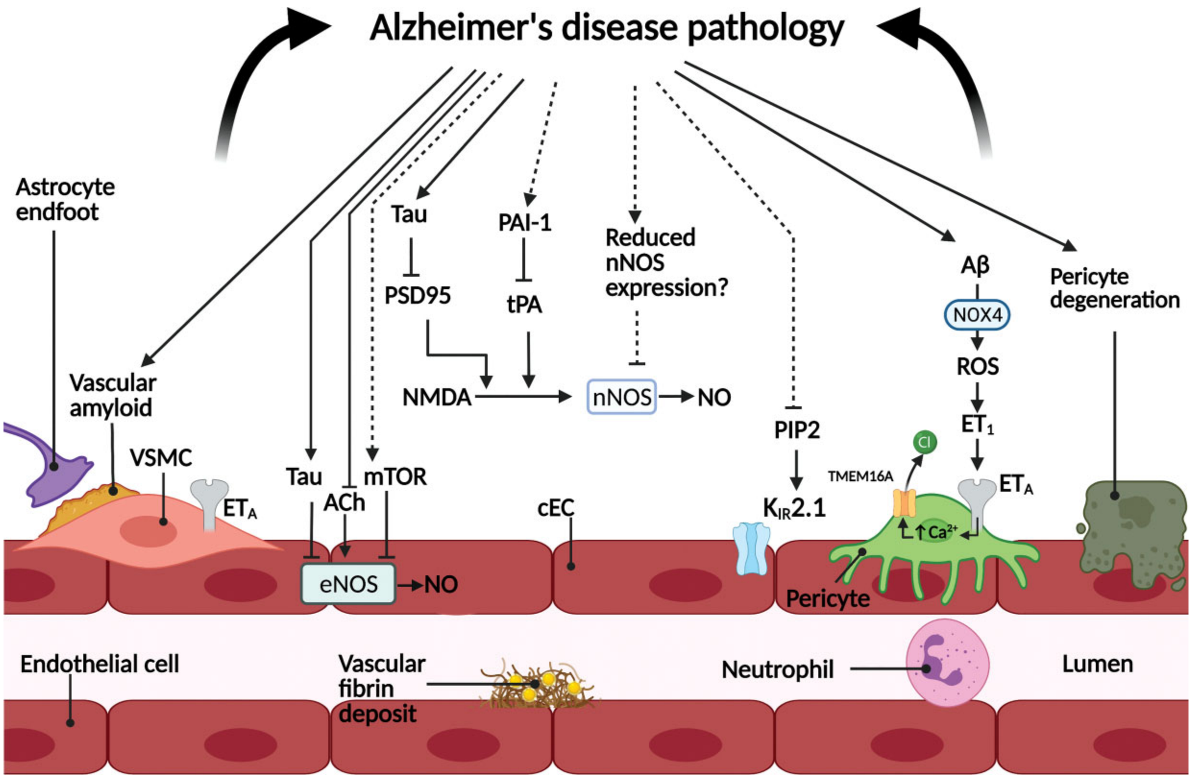

Figure 2 Neurovascular uncoupling in Alzheimer's disease. Schematic representing the current knowledge of Alzheimer's disease-associated neurovascular uncoupling. According to Zlokovic's two-hit hypothesis,[7] the contributions of these mechanisms to neurovascular dysfunction contributes to hit two (Aβ) in a feedforward cycle. Dashed lines represent pathways with limited or debated evidence. Created with BioRender.com. cECs = capillary endothelial cells; mTOR = mammalian target of rapamycin; PAI-1 = plasminogen inhibitor-1.

(iNOS) in Alzheimer's disease. eNOS and nNOS are constitutively expressed and are regulated by $[Ca^{2+}]_i$ signals, whereas iNOS is not regulated by $[Ca^{2+}]_i$ signals and is not constitutively expressed in cells.[127] iNOS expression has been linked to $A\beta_{1-40}$, peroxynitrite formation and poorer performance in cognitive tests in mice—an effect which is dependent on tumour necrosis factor-alpha (TNF-α).[128] Regarding the constitutively expressed NO synthases, a three-way comparison between biopsied brain tissue from 60-year-old neurologically normal donors, 80-year-old neurologically normal donors and 80-year-old donors with Alzheimer's disease showed that nNOS and eNOS expression were reduced in the frontal gyrus and hippocampus of Alzheimer's disease donors compared with both ages of neurologically normal donors.[129] This is interesting given it is established that these two brain regions are particularly vulnerable to Alzheimer's disease pathology.[130] Indeed, no difference in eNOS and nNOS expression was observed in the cerebellum between Alzheimer's disease donors and age-matched donors (although there was an age-related difference), which is in line with the observation that the cerebellum is usually affected in later stages of the disease.[130]

Recently, Nizari *et al*.[131] have investigated the link between loss of cholinergic innervation and reduced eNOS-dependent NVC in the mouse cortex and hippocampus. These authors found that saporin induces cholinergic denervation in both brain areas and causes a reduced CBF response to pharmacological stimulation of an eNOS-dependent pathway in the cortex but not the hippocampus. This finding corresponds with reduced eNOS expression in the cortex but not hippocampus of saporin-treated mice. This is a significant finding given the early cortical and hippocampal loss of cholinergic innervation in Alzheimer's disease.[132] Furthermore, Nizari *et al*.[131] provide evidence that reduced cholinergic innervation and eNOS-dependent vasomotility may predispose vessels to develop CAA.

Neuronal NOS expression and NO release in the Alzheimer's disease hippocampus is complex and appears heterogenous. In one study, Dias *et al*.[133] reported decreased glutamate induced NO release in CA1 hippocampal neurons of Alzheimer's disease mice. Conversely, the same group reported increased glutamate-induced NO release in the dentate gyrus of triple transgenic Alzheimer's disease mice, with an increase in nNOS density across the whole hippocampus compared with control mice.[134] In this latter study, the Alzheimer's disease mice exhibited impaired FH despite the increased NO release. This uncoupling between neuronal NO and hyperaemia may reflect a lower bioavailability of NO due to sequestration by ROS (forming peroxynitrites, a mediator of nitrosative stress).[135] Katusic and Austin make the point that increased ROS are a feature of Alzheimer's disease as well as the cardiovascular diseases that are risk factors for Alzheimer's disease;[136] thus, it is conceivable that this oxidative environment reduces the bioavailability of NO.

Recently, tau pathology has been shown to contribute to neurovascular uncoupling. Park *et al*.[137] have shown that mice overexpressing human tau (PS19 mice) have reduced NMDA-dependent NO release and diminished FH, effects which are seen before the onset of neurodegeneration or formation of tau tangles. The authors demonstrate that tau interferes with the action of PSD95, an anchoring protein that associates NMDA receptors with nNOS, without which NMDA activation fails to stimulate

Table 1 Studies of neurovascular uncoupling in Alzheimer's disease

Mechanism affected	Specific pathway	Evidence
Potassium ions		
	Reduced $K_{IR}2.1$ current	Mughal *et al.*[119] found a reduced $K_{IR}2.1$ current in Alzheimer's disease mice, secondary to a deficiency in the phospholipid PIP2
Nitric oxide		
	tPA deficiency	Park *et al.*[106] show that Alzheimer's disease mice are tPA-deficient and have attenuated NO release during FH
	Cholinergic denervation	Nizari *et al.*[131] show that cholinergic denervation leads to reduced eNOS-dependant NVC in the mouse cortex
	Tau pathology	Park *et al.*[137] demonstrate that tau interferes with NMDA–nNOS coupling in Alzheimer's disease mice resulting in reduced NVC. Van Skike *et al.*[138] report that tau inhibits NOS activation
	mTOR	Lin *et al.*[139] show that mTOR inhibits eNOS phosphorylation, leading to reduced cortical NO release. Van Skike *et al.*[141] report that rapamycin improves FH in Alzheimer's disease mice via NOS-dependant and independent pathways
Aβ		
	Exogenous Aβ application	Niwa *et al.*[125] show that Aβ application reduces CBF in mice. Nortley *et al.*[92] find that Aβ application causes capillary constriction in human brain slices
	Aβ depositions in human brain slices	Nortley *et al.*[92] correlated severity of Aβ deposition with capillary constriction at pericyte locations in human Alzheimer's disease brain slices
Pericyte loss		
	Single pericyte ablation	Hartmann *et al.*[75] use laser irradiation to ablate single pericytes, causing the associated capillary to dilate.
	PDGFRβ-deficient mice	Kisler *et al.*[70] report that *Pdgfrb*$^{+/-}$ mice have reduced pericyte coverage and NVC capacity
	Global pericyte KO	Kisler *et al.*[156] use Cre-recombinase to achieve acute global pericyte KO, leading to reduced NVC
Cerebrovascular amyloid deposits		
	Displacement of astrocyte endfeet and increased arterial stiffness	Van Veluw *et al.*[164] show that astrocyte-mediated NVC is disrupted in CAA affected vessels and that CAA-affected Alzheimer's disease mouse vessels may be less contractile
Vascular fibrin		
	Microvascular fibrin deposits interact with Aβ to become resistant to breakdown[171]	Cortes-Canteli *et al.*[170] showed that dabigatran, which inhibits fibrin formation, improves CBF in Alzheimer's disease mice
Neutrophil occlusion		
	Capillary occlusion by neutrophils	Cruz Hernández *et al.*[172] report that ~2% of brain capillaries are occluded by neutrophils in Alzheimer's disease mice

KO = knockout; mTOR = mammalian target of rapamycin; PDGFRβ = platelet-derived growth factor receptor beta.

NO production. Work published in poster form by Van Skike *et al.*[138] has corroborated the finding that tau suppresses nNOS and eNOS function, albeit by suppressing NOS phosphorylation rather than via PSD95. This study reports diminished hyperaemic response to whisker stimulation in PS19 mice based on laser Doppler flowmetry alone. Preserved neuronal activity must also be measured in order to attribute the change in hyperaemic response to a neurovascular uncoupling and not neuronal deficits. Thus, it is impossible to rule out the possibility that decreased neuronal activity, perhaps as a result of tau pathology, rather than neurovascular uncoupling leads to the observed reductions in FH. Electrocorticography and evoked field potentials are widely used in other studies to demonstrate preserved neuronal activity.[106,137]

It is thought that the mammalian target of rapamycin (mTOR) also contributes to the NVD in Alzheimer's disease by suppressing phosphorylation (at Ser1176) of eNOS, leading to diminished NO synthesis by the cortical endothelium.[139,140] Indeed, acute mTOR inhibition using rapamycin leads to Ser1176 phosphorylation of eNOS, increased NO release and vasodilation.[139] The same study also found that the reduction of CBF measurable in Alzheimer's disease mice is not present if the Alzheimer's disease mice are chronically administered rapamycin. Rapamycin-administered Alzheimer's disease mice also treated with the NOS inhibitor L-NAME show comparable CBF reductions to nonrapamycin Alzheimer's disease mice, providing support for rapamycin's effect via NOS. Recent work has extended this to show that rapamycin acts through both NOS-dependent and -independent mechanisms to improve NVC (even to levels above wild-type animals); importantly, the nNOS downregulation seen in their animal model precedes Aβ deposition, and reduced nNOS expression was seen even during early Alzheimer's disease changes based on Braak staging in human post-mortem samples.[141] Rapamycin was also found to restore global CBF reductions found in *APOE4* mice.[142] In a more recent fMRI study, it has also been shown that rapamycin restores the diminished fMRI signal in the somatosensory cortex upon forepaw stimulation seen in Alzheimer's disease mice.[143] However, as the authors note, these fMRI data cannot distinguish whether the rapamycin reverses neurovascular uncoupling or diminished neuronal activity.[143]

Amyloid-β and reactive oxygen species

In 2001, an *in vivo* study performed on mice demonstrated that topical application of Aβ reduced resting CBF.[125] This group also showed that *in vitro* Aβ application induces the constriction of mouse brain arteries. Taken together, these observations suggest that Aβ reduces CBF by inducing vasoconstriction. This effect was rescued by application of superoxide dismutase (SOD)—an enzyme that scavenges ROS—and was not observed when using a mutant strain of Aβ that does not induce ROS formation [$A\beta_{1\text{-}40}$(M35Nle)]. This points to ROS formation by Aβ as the mechanism for its vasoconstricting effects.

A more recent study by Nortley *et al*.[92] found that $A\beta_{1\text{-}42}$ constricts brain capillaries in biopsied human brain tissue. This observation was made using an acute application of exogenous Aβ. To explore an experimental paradigm more akin to chronic Aβ exposure in Alzheimer's disease, the authors studied human brain tissue from patients with and without Aβ depositions. The severity of Aβ deposition is likely to correlate closely with levels of vasoconstricting soluble oligomers with capillaries from patients with Aβ depositions found to be significantly more constricted than those without Aβ depositions. The authors found capillary constriction to be most pronounced at the pericyte soma. As this is where pericytes exhibit most contractile influence over capillaries, and considering the previous research confirming pericyte contractility,[18,21,144] it is apparent that pericytes mediate Aβ-induced vasoconstriction.[92] Extending their study to rat cortical slices, Nortley *et al*.[92] found that Aβ interacted with NADPH oxidase (NOX) 4 to form ROS—which in turn leads to release of ET-1 and activation of ET_A receptors—presumably on pericytes—leading to vasoconstriction. These mediators of Aβ-induced vasoconstriction were implicated because their specific pharmacological blockade abrogated the vasoconstriction caused by Aβ.[92] Importantly, it has previously been demonstrated that ET-1 is upregulated in temporal cortical neurons and microvessels in post-mortem samples from Alzheimer's disease patients.[145] As such, these findings have the potential for therapeutic benefit, but translation into human therapy remains distant and is complicated by the need for the drug to cross the BBB and act with specificity at capillary pericytes.[146] For example, resveratrol reduces NOX-dependent ROS formation in aged mice and improves NVC deficits,[147] and recent work has shown that inhibiting ROS formation reverses the hippocampal NVC deficit found in Alzheimer's disease mice *in vivo*.[148]

Recent work has identified the TMEM16A channel, a Ca^{2+}-activated chloride channel, as a mediator of pericyte depolarization and constriction.[149] It is hypothesized that a small rise in $[Ca^{2+}]_i$ (such as provoked by ET-1) could trigger chloride efflux via TMEM16A, depolarizing and constricting the pericyte. Supporting evidence comes from experiments showing that pharmacological inhibition as well as genetic knockout of TMEM16A channels reduces ET-1-evoked vasoconstriction, and that reducing $[Cl^-]_i$ abolishes this effect. Given that TMEM16A inhibition blocks pericyte contraction and thus improves CBF in an *in vivo* mouse model of stroke,[149] future work should aim to elucidate if TMEM16A plays a role in NVC and if its blockade can prevent the NVC deficits caused by Aβ-dependent ET-1 release mentioned above.[92]

Physical mechanisms of neurovascular uncoupling

Pericyte loss

An Alzheimer's disease-associated decrease in pericyte coverage has been found in post-mortem human biopsies of the hippocampus,[150] cortex[150,151] (although a recent study found no loss of frontal cortical pericytes compared to similar aged controls[152]) and retina.[153] There is also convincing mechanistic evidence: a very recent single-cell transcriptomic study has demonstrated that 30 of the top 45 Alzheimer's disease vulnerability genes are expressed in the microvasculature, and that there appeared to be a selective loss of pericyte subtypes that were associated with BBB maintenance, as well as impairment in signalling pathways associated with CBF regulation.[154] At 1–2 months of age *Pdgfrb*$^{+/-}$ mice exhibit moderate loss of brain capillary pericyte coverage[70,155] but preserved neuronal activity, and are thus useful in the study of pericyte loss in NVC. Indeed, these mice exhibit a 30% reduction in hyperaemic response to hindpaw stimulation compared to *Pdgfrb*$^{+/+}$ mice, suggesting pericyte loss leads to neurovascular uncoupling.[70] Similarly, neurovascular uncoupling was also observed when global pericyte loss is achieved acutely using a Cre-recombinase-based technique.[156] Single pericyte ablation can be achieved using laser irradiation and allows the study of pericyte loss without the confounding factors of BBB dysfunction.[75,157] This leads to dilation in the capillary associated with the targeted pericyte and increased red blood cell velocity and flux compared to sham ablation (laser irradiation at sites along the endothelium but not at pericyte locations).[75] This vasodilation may contribute to disruption of optimal capillary flow.[75,158]

Even when any flow reduction (in the resting state or during activity) is insufficient to cause direct ischaemic injury, there are intriguing hypotheses that capillary flow dysregulation can have deleterious effects even when total CBF is unaltered. Upstream dilation without a corresponding increase in the uniformity of capillary flow, a phenomenon referred to as CTTH, could impair or even invert the actual delivery of oxygen to the parenchyma due to an overall reduction in oxygen extraction.[158] Initially described in modelling studies,[159] CTTH and capillary hypoperfusion have now been demonstrated in dynamic susceptibility contrast studies of Alzheimer's disease patients[96] and, crucially, in patients with mild cognitive impairment,[97] suggesting it is an early feature of Alzheimer's disease rather than just a late consequence of neuronal death.

Cerebrovascular amyloid deposits

CAA is the deposition of amyloid in capillary and arteriolar walls of the cerebral vasculature and affects the majority of Alzheimer's disease patients.[160,161] Mouse models of Alzheimer's disease-related CAA show impaired hyperaemic response to various vasodilatory stimuli[162] as well as capillary occlusion and CBF disturbances.[163] This relationship may also be bidirectional, with loss of NVC impairing clearance of Aβ and predisposing to CAA.[131] However, results from mouse models of CAA are likely confounded by the presence of other pathologies, such as parenchymal Aβ plaques.[162] Although both forms of plaques coexist in Alzheimer's disease brains, this nonetheless makes it difficult to isolate the effects of CAA on neurovascular uncoupling.

Van Veluw and colleagues[164] were able to show *in vivo* that awake mice with CAA have impaired vasodilatory response to visual stimulation. Interestingly, no association was found between vessel CAA load and vasoreactivity—leading the authors to instead hypothesize that VSMC loss in CAA-affected vessels leads to the impaired vasoreactivity. Another mechanism by which CAA may lead to neurovascular uncoupling is by displacement of astrocyte endfeet from arterioles, as shown by imaging studies, which may disrupt astrocytic control of vessel diameter.[165] Indeed, in

Alzheimer's disease mice, targeted release of caged Ca^{2+} in astrocytes caused reduced constriction in vessels affected by CAA, whereas vessels without CAA showed constriction comparable to control mice. In Alzheimer's disease mice, *in vivo* laser stimulation of VSMCs exhibited reduced vasoconstriction in cells of arterioles with vascular amyloid burden, but not those free from vascular amyloid burden. In fact, Alzheimer's disease mouse VSMCs associated with no vascular amyloid burden exhibited comparable vasoconstriction upon laser stimulation to VSMCs in control mice. This led the authors to conclude that CAA not only disrupts the astrocyte–VSMC interaction but also causes arterial stiffness leading to reduced vasomotility.[165]

Blood–brain barrier breakdown

A recent MRI study demonstrated gradual BBB breakdown during healthy ageing, which was worst in the hippocampus, corresponding to known vulnerability in Alzheimer's disease; importantly, this was accelerated in cognitive impairment, and the degree of BBB breakdown correlated with pericyte loss based on CSF biomarkers.[87] This validated a large body of work supporting pericyte loss and subsequent BBB dysfunction as a key step in driving neuronal loss and Alzheimer's disease-like pathology.[155,166] There are myriad proposed mechanisms of how loss of BBB integrity contributes to neuronal death, which are reviewed in depth elsewhere,[167] but ultimately lead to excess neurotoxic agents, either through direct leakage from the circulation, or local production as part of an inflammatory response.

One example of a potentially neurotoxic mediator is fibrin, a plasma protein that normally cannot cross the BBB. *APOE4* is associated with pericyte damage and BBB breakdown.[87,168] Human *APOE4* carriers (heterozygotes and homozygotes) were found to have selective hippocampal and medial temporal gyrus BBB breakdown, which correlated with cognitive impairment.[168] *APOE4* carriers exhibit approximately seven times more extravascular fibrin deposits than *APOE3* carriers.[151] Fibrin itself contributes to Alzheimer's disease pathogenesis, both as a result of leakage into the brain parenchyma through a leaky BBB[169] and accumulation in brain microvessels in response to vascular injury.[170] This latter effect is worsened by Aβ, which interacts with fibrin clots to make them resistant to breakdown.[171] Cortes-Canteli *et al.*[170] found that dabigatran, which inhibits the conversion of fibrinogen to fibrin by thrombin, reduces soluble and insoluble Aβ species and improves cognitive function in Alzheimer's disease mouse models. These effects may be secondary to the increase in CBF observed in Alzheimer's disease mice treated with dabigatran compared to controls.

Neutrophil occlusion

Cruz Hernández *et al.*[172] observed using two-photon electron microscopy that ∼2% of capillaries become occluded by neutrophils in APP/PS1 mice (an Alzheimer's disease model with especially elevated $A\beta_{1-42}$ levels), that certain capillaries were more likely to become occluded than others and that these occluded capillaries were narrower than nonoccluded capillaries. The finding from Nortley *et al.*[92] that $A\beta_{1-42}$ causes pericytes to constrict capillaries provides a possible mechanism for the observed reduction in capillary diameter found in APP/PS1 mice.[173] These capillaries could belong to the subset of capillaries that are associated with pericytes expressing αSMA, which focally constrict and thus are more likely to become occluded by neutrophils.[92] This demonstrates the interplay between aberrant signalling and physical obstruction: aberrant signalling mediated by Aβ ultimately leads to ET release and pericyte constriction, which predisposes the affected capillaries to occlusion by neutrophils.

Conclusion

A future challenge of the field is how NVC mechanisms can be manipulated therapeutically for the treatment of the NVD that underpins neurodegenerative diseases such as Alzheimer's disease. Future studies should mainly focus on *in vivo* experiments where possible, as meta-analysis suggests that these results show most validity between studies.[13] While *in vitro* studies are generally limited by use of preconstricting agents and electrical stimuli,[13,51] these studies may still be used effectively to complement *in vivo* findings.[92] Study of the various mechanisms of neurovascular uncoupling is underway and has begun to link changes in Alzheimer's disease to diminished FH, identifying possible therapeutic targets along the way. One caveat to this is that the physiological stress of experimental preparation may confound findings, and that at least one model of Alzheimer's disease (J20) may be more sensitive to these stresses than control mice.[174] On the one hand, Aβ and tau may both contribute to neurovascular uncoupling, and on the other, neurovascular uncoupling may promote hypoxia, which facilitates Aβ accumulation. Thus, the spatiotemporal relationship between brain regions vulnerable to Alzheimer's disease pathology and neurovascular changes remains a key unanswered question with implications for disease pathogenesis and treatment strategies.

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